Jozef J. Bujarski
Department of Biological Sciences
Northern Illinois University
DeKalb, IL 60115
Email: T80jjb1@wpo.cso.niu.edu

Final Progress Report

Biotechnology Risk Assessment Research Grants Program
Proposal No. 9601416
Award No. 96-39210-3842
Title: Recombination of Viral and Transgene RNAs in Susceptible and Resistant Plants.

I. Specific Aims/Objectives

There are three objectives for this project: (i) to study recombination of brome mosaic virus (BMV) during double infection in non-transgenic plants; (ii) to study recombination between viral transgenes and infectious virus (low selection pressure), and (iii) to study recombination between viral transgenes and non-infectious virus (high selection pressure). In the first year of this project we concentrated on objectives (i) and (ii). For objective (i) both systemic (*Nicotiana benthamiana*) and local lesion (*Chenopodium quinoa*) hosts were singly or doubly infected with BMV variants having marker mutations within the RNA3 component and the progeny RNA was analyzed for the recombinant content. For objective (ii) constructs with the desired portions of BMV RNA3 that were suitable for transformation of *Nicotiana benthamiana* plants were obtained.

In the second year, we have concentrated on objectives (ii) and (iii). In collaboration with Monsanto Company, the heterozygous transgenic plants of *N. benthamiana* that express five different partial BMV RNA3 constructs (containing the coat protein (CP) gene) were generated, and tested for the CP-mediated resistance against BMV infection. Subsequently, the susceptible lines were infected with either infectious or non-infectious BMV RNA3 constructs (plus the wt RNAs 1 and 2), and the levels of infection and recombination were determined by the DAS-ELISA.

In addition, a portion of the grant funds was used to study the mechanism of recombination in BMV (see the first three publications cited at the end of this report).

II. Results and Discussion.

Results of objective (i): Recombination under low selection pressure: double infection of non-transgenic plants. In order to determine recombination frequencies under low selection pressure during double infection of non-transgenic plants, we created four noncompetitive mutants of the BMV RNA3. The non-transgenic plants used were *Chenopodium quinoa* that was a local-lesion host for BMV. These plants were inoculated with infectious transcripts of BMV RNA1 and 2 and mutated 3. Each RNA3 mutant contained a silent mutation, which did not affect the two encoded proteins (3a and CP), but did introduce (or eliminate) a restriction site in the corresponding DNA sequence of RNA3. These mutations were : (i) A replacing a T at position 121 creating a BamH1 site; (ii) insertion of a 3x(GATC) at position 860, which eliminated a BclI site; (iii) insertion of TT at position 1318 and insertion of a T in position 1321 creating of a BamHI site with mutation (ii); and (iv) an insertion of AT in position 1862 creating of a BamH1 site. All the constructs

were cloned into pB3TP7 (the transcribable wt RNA3 cDNA clone), and the infectious transcripts were synthesized using a Megascript kit from Ambion.

The mutated RNA3 transcripts were mechanically inoculated on two-week-old *C. quinoa* plants (together with wild-type transcripts of RNAs1 and 2), at a concentration of 1 μg/ml each. Local lesions were collected two weeks after inoculation and the viral RNA was extracted. After RT-PCR amplification of these RNA, the cDNA was analyzed by restriction analysis, cloning and sequencing.

The results from the singly inoculated plants showed that the four mutated RNAs3 were infectious, and were stable on both systemic and local lesion host plants. The viral progeny of one systemic plant for each RNA3 mutants and of 8, 6, 8 and 8 local lesions for each RNA3 mutant, respectively, revealed the same restriction maps as their respective parental RNA3 inocula. This indicated that the four mutations introduced in each RNA3 mutant did not affect their ability to replicate and to be encapsidated.

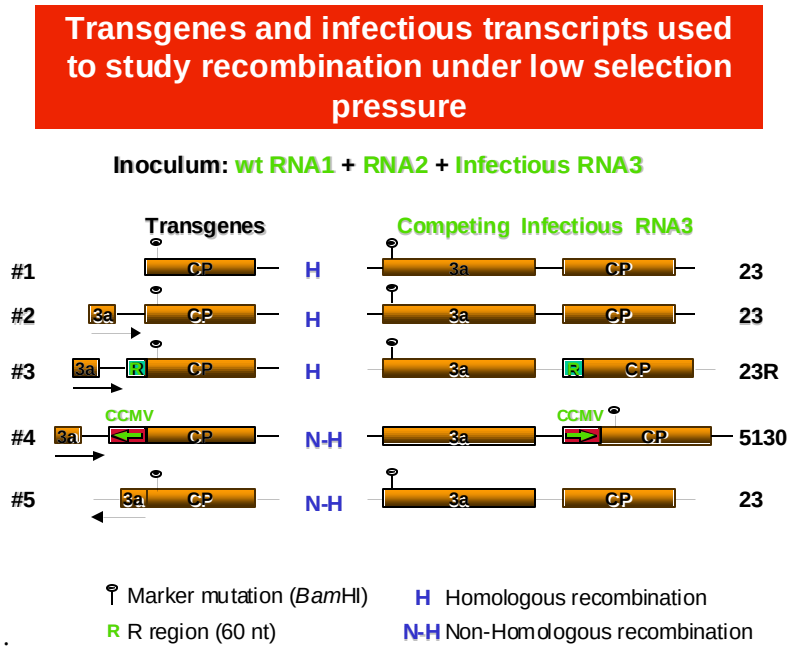
The results from doubly inoculated (with a combination of two RNA3 mutants) plants were obtained from three independent experiments. In the first experiment, no recombinants were obtained. Each local lesion (5 for each combination of RNA3 mutants) contained only one parental RNA3 mutant, supporting the theory that in order to observe recombinant RNA3, the recombination event should happen at the initial stage of viral infection. For both combinations, a similar number of local lesions was induced by either RNA3 mutant, suggesting that these mutants behaved alike. In the second experiment, most local lesions contained one or the other parental RNA 3 variant. However, some of the lesions contained a combination of the two introduced marker mutations, demonstrating that the two different inoculated RNAs3 can co-replicate in the same local lesion. Cloning and sequencing of progeny RNA3 have demonstrated the high recombination frequencies in doubly-infected local lesions, and revealed the existence of a homologous recombination hot spot within the intergenic region. The intergenic region on BMV RNA3 comprises the subgenomic RNA4 promoter and a 1a protein binding site. We are now in process of introducing marker mutations into the subgenomic region to further elucidate the role of the intergenic sequences in supporting homologous crossovers.

Our data suggest that the double infection system is useful in determination of homologous recombination frequencies especially that neither of the marker mutations used have displayed any selective advantage during infection. These results are published in J. of Virology (see below under Bruyere et al., J. Virol. 74, 4214-4219).

Results for objectives 2 and 3: Construction and testing of transgenic* Nicotiana benthamiana *plants expressing BMV RNA3 sequences.

As planned in our initial grant application, five different constructs suitable for transformation of *N. benthamiana* plants were obtained (Fig. 1). These transgenes contained: #1, the complete CP gene and the 3'UTR (nt 1235-2117 of BMV RNA3); #2 and #5, the CP gene together with the intercistronic region of BMV RNA3 in positive (nt 860-2217) or reverse orientation (nt 1225-860/1226-2217), respectively; #4, the same construct as #2 plus a RNA3 CCMV insert of 158nt (nt 992-1149) inserted at position 1253 of BMV RNA3; and #3, the same as #2 with an RNA3-derived recombinationally-active region R (a 60 nt (nts 1899-1958) insert at position 1253 of BMV RNA3. All the constructs were derived from a full length cDNA clone of BMV RNA3 (designated pB3TP7, kindly provided by Alhquist laboratory) and introduced into the transformation vector pMon11781 from Monsanto Co. All these transgenes were ligated downstream of a strong transcription promoter derived from the 35S promoter from the figwort mosaic virus. The pMON 11781 vector also contained the NPT-II gene, as a selectable marker.

A.



B.

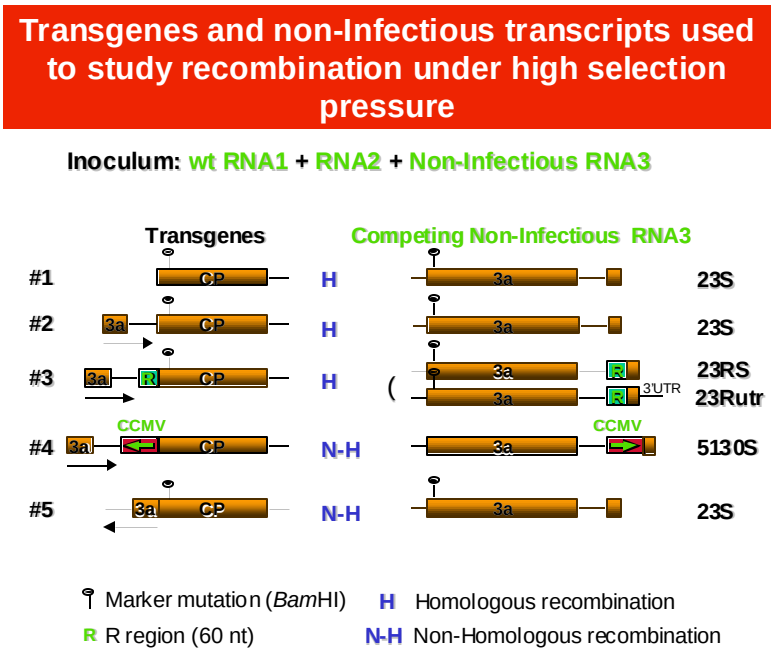


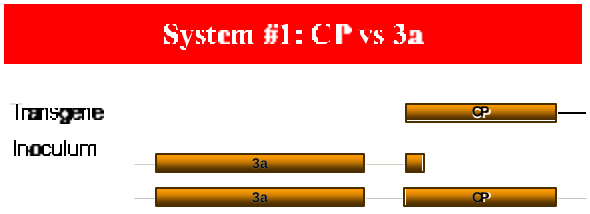
Figure 1. Diagrammatic representation of BMV RNA3 constructs used in these studies as transgenes (shown on the left side) and as the inocula (shown on the right side). Panel A shows the transgenes and the infectious transcripts used to study recombination under low selection pressure, whereas panel B shows the same for high selection pressure.

The transformation experiments were performed at Monsanto Co. Monsanto provided us 20 RO lines of transgenic *N. benthamiana* plants per each construct. These lines were first grouped according to their resistance against wt BMV infection. Plants were inoculated with *in vitro*-transcribed BMV RNAs 1 and 2 , and the corresponding modified RNAs3. For constructs #1, #2, #3, #4, and #5 the following percentages of transgenic lines were found to be susceptible towards the BMV infection, respectively: 21%, 47%, 59%, 45%, 58%, and 63%. From these experiments, we concluded that construct #1 induced the anti-BMV resistance in the highest number of lines.

Thereafter, the transgenic lines were tested for the expression of the marker NPTII gene. Since the NPTII marker was the integral part of the transgene constructs, its expression reflected the levels of expression of the BMV transgene sequences. These experiments were performed to find out whether there was correlation between the expression of transgenes and the susceptibility to BMV infection. The DAS-ELISA assays revealed that the highest NPTII expressors were resistant against BMV infection, whereas the moderate, and especially the lowest expressors, were susceptible to BMV. However, in some lines this correlation was not observed (i.e., low NPTII expressors did not support BMV infection). We concluded that the expression of BMV sequences usually prohibited BMV infections but in some RO plants the transgene probably hit the host functions, that were essential for BMV infection.

The selected R0 lines per each transgene construct (#1 to #5) were micro-propagated and 20 to 30 seedlings (per line) were inoculated with either infectious or non-infectious corresponding RNA3 constructs, in order to determine recombination frequencies under low and high selection pressure. The establishment of viral infection was tested by the DAS-ELISA. The following observations were made (see Fig. 2, below):

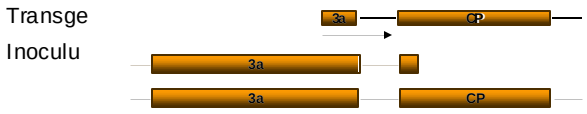
A.



Line	BMV resistance	% of positive plants in ELISA	
		Low Selection	High Selection
260	No	15	0
270	No	26	0
264	Yes	12	4
273	Yes	6	6

B.

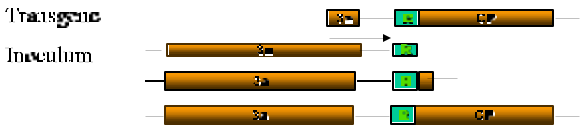
System #2: CP and 3a (longer overlap)



Line	BMV resistance	% of positive plants in ELISA	
		Low Selection	High Selection
282	No	13	4
283	No	30	10
287	No	25	16
285	Yes	11	12
286	Yes	17	9
290	Yes	32	7

C.

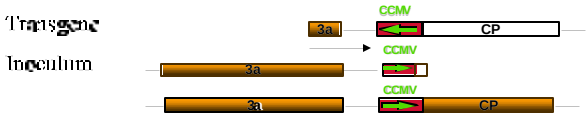
System #3: CP-R and 3a-R (3'-UTR)



Line	BMV resistance	% of positive plants in ELISA	
		Low Selection	High Selection
319	No	8	0
323	No	0	27 (25)
331	No	8	8 (13)
324	Yes	9	0 (0)
325	Yes	0	0
330	Yes	22	0 (75)

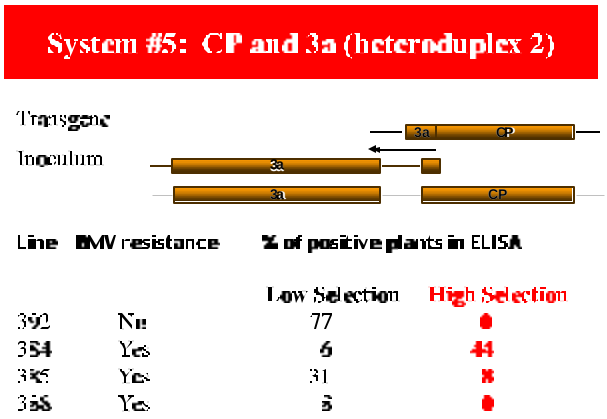
D.

System #4: CP and 3a (CCMV heteroduplex)



Line	BMV resistance	% of positive plants in ELISA	
		Low Selection	High Selection
301	No	12	0
313	No	33	8
309	Yes	36	0
307	Yes	50	0

E.



F.

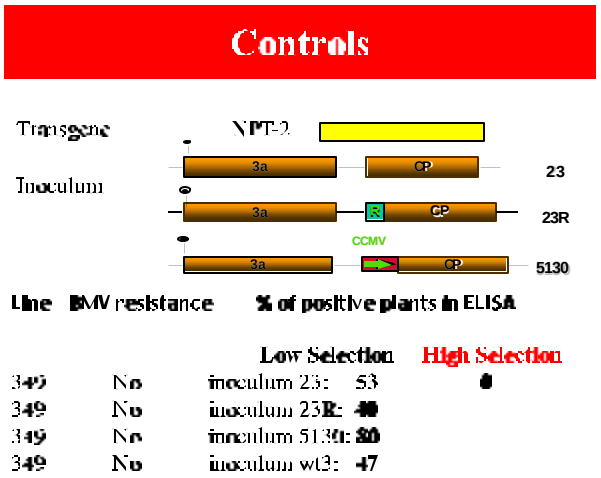


Figure 2. Diagram summarizing the results of inoculation of transgenic *N. benthamiana* plants with two types of inocula: the truncated (noninfectious) versions of RNA3 (for high selection pressure experiments) and full-length (infectious) versions of RNA3 (for low selection pressure experiments). Each transgenic plant system is shown in a separate panel, and the percentages of plants (positive in DAS-ELISA) that developed BMV infection, are shown below in each panel. Typically between 20 and 40 micropropagated seedlings were inoculated per each line.

In system #1 (Fig. 2A), the CP transgene and the truncated non-replicative RNA3 inoculum have overlapped within the CP sequence. Interestingly, the inoculation with this truncated RNA3 construct developed infection (i.e., generated recombinants) in a small fraction of plants from two BMV-resistant lines (lines 264 and 273) but no infection (i.e., no recombinants) were induced in plants of two BMV-susceptible lines (lines 260 and 270). Much higher fractions of plants for each tested lines have developed BMV infection after inoculation with the full-length RNA3 construct. This suggested that the overlapping sequences could promote RNA recombination events. However, no correlation between anti-BMV resistance and recombination was observed.

For system #2, where CP and 3a RNA3 components overlapped through almost 400 nucleotides, the inoculation with full-length RNA3 (low selection pressure) induced infection in a higher number of plants, that were not BMV resistant, than in those that were BMV resistant (Fig. 2B). The inoculation with truncated RNA3 construct induced viral infection in both types of plants. These results demonstrated that the overlapping intercistronic sequence (of RNA3)

supported the RNA recombination, and that there was no clear correlation between anti-viral resistance and recombination.

The results of the DAS-ELISA assays were related to the background levels of the BMV CP expression in uninfected transgenic plants. Typically, the establishment of BMV infection increased the BMV CP concentrations up to least five-fold.

For system #3, which carried the recombinationally active region R, the use of the low selection (full-length) RNA3 construct induced viral infection in a much lower number of seedlings, as compared to the use of the truncated (high selection pressure) RNA3 construct (Fig. 2C). In contrast, system #4 carried a complementary CCMV insert within the intercistronic region of RNA3. Here, more lines were infected with the full-length RNA3 than with the corresponding truncated RNA3 (Fig. 2D). Apparently, the overlapping region R supported much higher recombination frequency than the tested regions of complementarity between the RNAs. It supported previous observations that homologous recombination events were much more frequent than the nonhomologous recombination events.

In system #5, the transgene contained the intercistronic region in a reverse orientation as compared to the wt BMV RNA3 (Fig. 2E). Inoculation with the upstream RNA3 fragment, that contained the intercistronic region complementary to the transgene (high selection pressure) induced viral infection (i.e., created recombinants) in some lines but not in others. The infection was induced in a higher number of plants while inoculating with the full-length wt RNA3 (low selection pressure). There was no clear correlation between anti-BMV resistance and the infectivity.

Control experiments were performed on transgenic plants that expressed only the NPT-II gene. The micropropagated seedlings of RO lines were inoculated with full-length BMV RNA3 constructs, including the wt RNA3, the RNA3 carrying the internal region R and the RNA3 with an internal CCMV sequence (Fig. 2F). None of the used lines were anti-BMV resistant, and a high number of micropropagated plants have established the BMV infection. This demonstrated that the expression of the NTP-II gene *per se* did not inhibit the BMV infection.

III. Conclusions

1. Recombination can occur between truncated RNA3 inoculum (high selection pressure) and transgenes in both BMV-resistant and BMV-non-resistant plants.
2. Recombination is also possible under low selection pressure, but to a lesser extent.
3. The length of the overlapping sequence between recombining RNAs seems to be important.
4. Region R does not necessarily support homologous recombination with transgenes (when taken out from its natural sequence context in wt RNA3).
5. Homology-driven recombination is more efficient than the heteroduplex-driven recombination, which confirms our previous observations.

IV. Follow-up Studies

For objective (i): We are continuing analyses of the role of intergenic promoter in homologous and nonhomologous recombination by using doubly infected systemic and local lesion host plants. At this moment our studies concentrate on the BMV system, and we are using an RNA3 construct that carries a duplication of the intergenic region. Such construct allows us to introduce of marker mutations (or other sequence modifications), in order to map the particular intergenic sequences that are responsible for recombination.

For objectives (ii) and (iii): We are in the process of generating homozygous lines of selected transgenic *N. benthamiana* plants. Thereafter, the plants will be again inoculated with the testing BMV RNA constructs (as of Fig. 1), in order to determine the effect of gene dosage on BMV RNA recombination, under low and high selection pressure.

V. Publications

Nagy, P., and Bujarski, J.J. (1998) Silencing homologous RNA recombination hot spots with GC-rich sequences in brome mosaic virus. J. Virol. 72, 1122-1130.

Figlerowicz, M., Nagy, P., Tang, N., Kao, C.C., and J.J. Bujarski. (1998) Mutations in the N-terminal domain of the brome mosaic virus RNA polymerase affect homologous and nonhomologous RNA recombination. J. Virology 72, 9192-9200.

Nagy, P.D., Ogiela, C., and J. J. Bujarski (1999) Mapping sequences active in homologous RNA recombination in brome mosaic virus: prediction of recombination hot spots. Virology 254, 92-104.

Bruyere, A., Wantroba, M., Flasinski, S., Dzianott, A., and J. J. Bujarski. (2000) Frequent homologous recombination events between molecules of one RNA component in a multipartite RNA virus. J. Virology 74, 4214-4219.